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Advances in Brief

Isolation and Identification of Fresh Tumor-derived Endothelial Cells from a Murine RIF-1 Fibrosarcoma[1]

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Abstract

Tumor vasculature is anatomically heterogeneous and distinct from the vasculature found in normal mature tissues. Examination of the differences between tumor and normal vasculature is critical to the future design of therapeutic modalities which either target tumor vasculature or potentially enable more efficient delivery of tumor cytotoxic agents. Such efforts to date have been hampered due to the inability to isolate live endothelial cells from solid tumors. We report here the isolation of fresh, noncultured endothelial cells from a C3H/HeJ RIF-1 murine fibrosarcoma through the use of fluorescence-activated cell sorting based on antibody staining for angiotensin-converting enzyme with further characterization by uptake and metabolism of acetylated low-density lipoprotein, factor VIII staining, and electron microscopy.

Introduction

Tumor development beyond 1–2 mm is dependent on the formation of a functional blood supply system for nutrient delivery (1). Based on studies involving intact vessels or established cell lines, tumor and normal blood vessels differ in their permeability, composition of the basement membrane, or extracellular matrix and cellular composition. When compared to normal, tumor vessels are tortuous, poorly organized, and hyperpermeable or leaky to circulating macromolecules (2–4). Dvorak *et al.* (5) have demonstrated that only mature vessels around tumor nodules and at the tumor-host interface leak significantly, while more immature tumor-penetrating vessels did not. However, the basement membrane is significantly reduced in new tumor vessels when compared to normal, thereby contributing architecturally to the leaky nature of tumor vessels (6). In addition, tumor vessels are composed primarily of a single layer of endothelial cells with normal vessels containing vascular pericytes which grow adjacent to the endothelial cells on the exterior of the vessel (7). The cell surface glycoprotein, endosialin, is selectively expressed in vascular endothelial cells as detected by immunohistochemical analysis of fixed tissue sections from human tumors (8). Studies to elucidate further these differences with potential significance for innovative therapeutic intervention of tumor vascular targeting (9, 10) have been hampered due to the lack of readily available and purified tumor-derived vascular endothelial cells for analysis.

Routinely used markers for endothelial cells include the presence of factor VIII-related antigen (11, 12), angiotensin-converting enzyme (13), and the accelerated uptake and metabolism of ac-LDL[3] (14, 15). As a result, we utilized FACS and the monoclonal antibody against ACE which binds to murine, bovine, and human endothelial cells (16) to develop a method where sufficient numbers of endothelial cells could be isolated from a RIF-1 murine fibrosarcoma with relative purity and little or no cell culturing or passage.

Materials and Methods

Tumor Model System. The RIF-1 fibrosarcoma model was maintained as previously described (17) in 6- to 10-week-old female C3H/HeJ mice obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were regularly monitored for the absence of adventitious murine viruses, and were housed and cared for in accordance with institution guidelines. RIF-1 tumors were produced by s.c. inoculation into the right flank with 10^5 log-phase tissue culture cells. Tumors were removed for study 14–16 days later when they had reached a weight of 0.5–0.7 g.

Tumor-derived Endothelial Cell Isolation. Tumors were aseptically removed and placed in cold Hanks' balanced salt solution with 50 units/ml heparin. Peripheral and necrotic tissues were excised and remaining tumor was

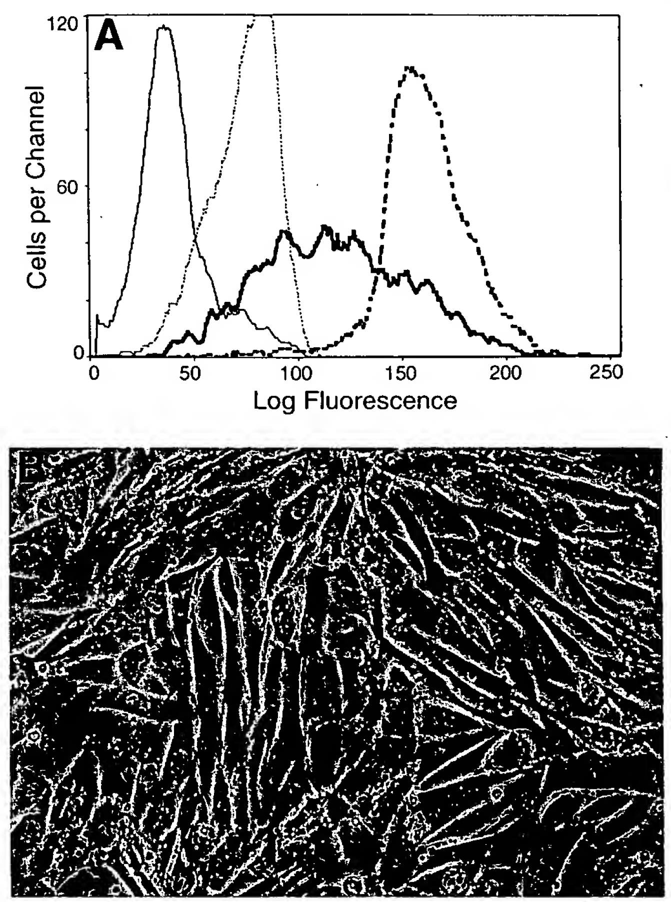

Fig. 1. Endothelial cell binding of antibody to ACE through the use of FACS. *A*, histogram of unlabeled (——), labeled unsorted (▬▬), labeled negative sorted (·····), and labeled positive sorted (- - - -), population of dissociated RIF-1 tumor. *B*, photomicrograph of sorted ACE-positive cells.

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[3] The abbreviations used are: ac-LDL, acetylated low-density lipoprotein; FACS, fluorescence-activated cell sorting; ACE, angiotensin-converting enzyme; DiI-ac-LDL, acetylated low-density lipoprotein, 1,1′-dioctadecyl-3,3,3′,3-tetramethyl-indocarbocyanine perchlorate; DMEM, Dulbecco's minimal essential medium; PBS, phosphate-buffered saline.

minced by using a scalpel. Dissociation of 0.5 g minced tissue was performed in a cold enzyme cocktail of 5 mg collagenase type II, 5 mg collagenase type IV, 10 mg DNase, 5 ml DMEM, 0.2 ml NuSerum (Collaborative Research, Lexington, MA) for 45 min of constant mixing. The cell suspension was passed through gauze, Hanks' balanced salt solution washed, then washed with PBS without Ca^{2+} or Mg^{2+}. Cells were suspended in FACS buffer (0.5% bovine serum albumin in PBS without Ca^{2+} or Mg^{2+}) at 1×10^6 cells/ml, and labeled with a 1:100 dilution monoclonal antibody to rat ACE. Two aliquots of tumor cell suspension were also labeled with a null antibody [F(ab')$_2$ fragment] or no primary antibody. Cells were incubated at room temperature for 30–40 min, followed by two washes with FACS buffer. Cold reagents were utilized to minimize antibody capping. Cells were suspended as before, then labeled with the secondary antibody: fluorescein isothiocyanate-conjugated goat anti-mouse IgM μ chain specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:400 dilution for 30–40 min. Following incubation, cells were washed twice with FACS buffer, suspended in FACS buffer at 3.5×10^6 cells/ml, and stored on ice until transported for sorting. Cells were sorted on a Becton Dickinson FACStar equipped with an argon ion laser set at 488 nM excitation wavelength. Positive sort cells were directed into tubes containing 20% FBS in DMEM with penicillin/streptomycin. Sorted cells were stored on ice until culturing.

Sorted cells were plated onto 1% gelatin- and media-conditioned 25-cm^3 plastic tissue culture dishes (Corning, Corning, NY) and were designated passage 0. Growth medium was DMEM enriched with 20% Sarcoma 180-conditioned media (18), 10% fetal bovine serum, 5 units/ml heparin, 1.34 mM L-glutamine, Eagle's basal medium with vitamins, and 50 μg/ml endothelial cell growth supplement (Collaborative Research).

Uptake and Metabolism of ac-LDL. ACE-positive cells were sorted based upon uptake of the fluorescent probe of acetylated-low density lipoprotein, DiI-ac-LDL (Biomedical Technologies, Inc., Stoughton, MA). Cells were labeled as described previously (11) with the same control cells as utilized for

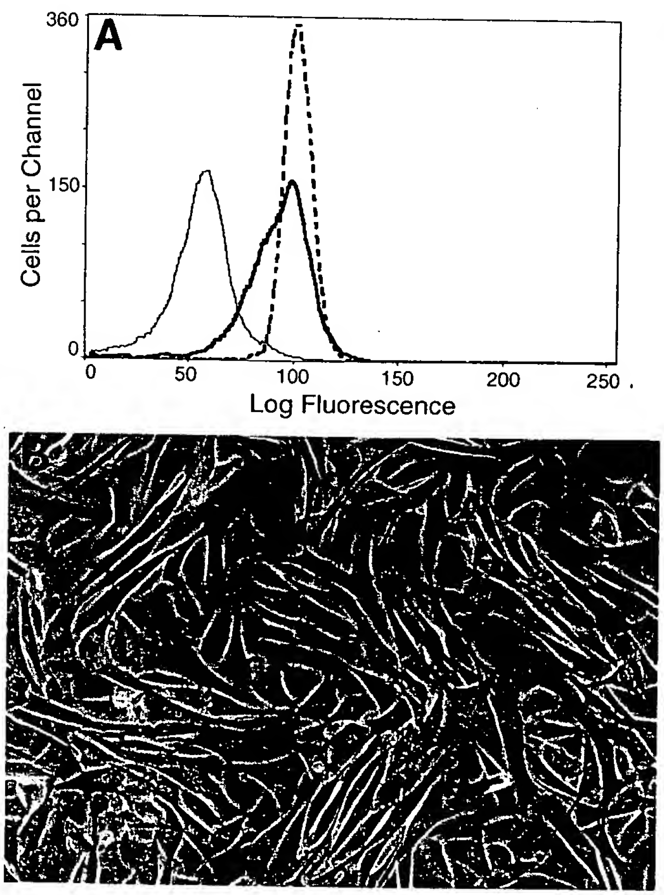

Fig. 2. Uptake of DiI-ac-LDL by ACE-positive cells through the use of FACS. *A*, histogram of unlabeled (——), labeled unsorted (——), and labeled sorted (- - - -) population of ACE/DiI-ac-LDL-positive cells, *B*, photomicrograph of sorted ACE/DiI-ac-LDL-positive cells.

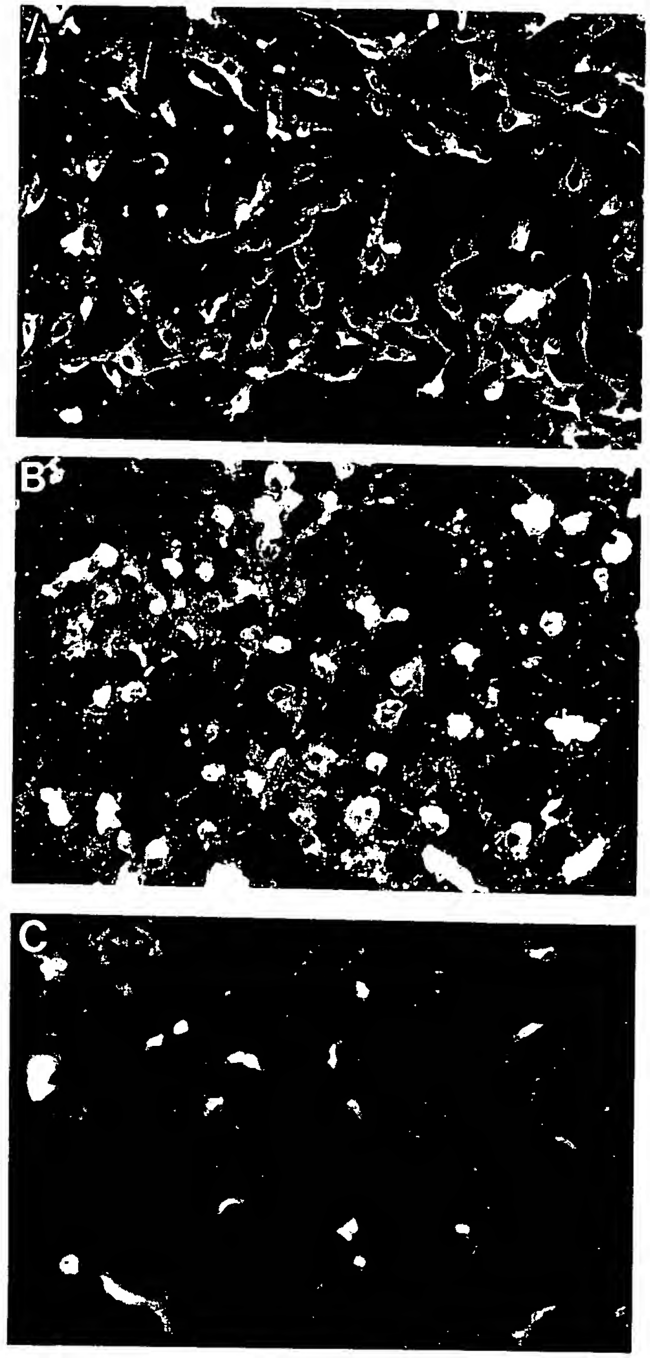

Fig. 3. Factor VIII staining of tumor-derived endothelial cells. Factor VIII stained *A*, sorted ACE/DiI-ac-LDL-positive cells; *B*, positive control hemangioendothelioma cells; and *C*, RIF-1 tumor cells, ×200.

ACE. Briefly, cells incubated with DiI-ac-LDL were washed twice with label-free media, then trypsinized to a single cell suspension in FACS buffer and FACS analyzed on excitation wavelength of 585 nm with a 42-band pass.

Factor VIII Staining. Factor VIII staining was by means of a double antibody-labeling method. Cells were grown on glass coverslips, fixed with cold methanol, blocked with 0.5% bovine serum albumin, labeled with antisera to factor VIII-related antigen (goat anti-human; ICN Biomedicals, Inc., Irvine, CA) at a 1:40 dilution, then stained with 1:50 dilution of fluorescein isothiocyanate-conjugated rabbit anti-goat IgG, or alternatively, with the control primary antibody, nonspecific F(ab')$_2$ fragment (both antibodies, Jackson ImmunoResearch Laboratories, Inc.).

Electron Microscopy. Cells were grown to confluence in 35-mm dishes, washed in PBS, fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide containing 0.1% potassium ferricyanide, dehydrated through graded alcohols, and embedded in Epon (Energy Beam Sciences, Agawam, MA).

Fig. 4. Electron photomicrograph of sorted ACE/DiI-ac-LDL-positive tumor-derived endothelial cells. Cell junctions (*arrows*) and microfilaments (*arrowheads*) are defined, ×35,000. Weibel-Palade bodies were also apparent, *Inset*, ×35,000.

En face sections were cut (50–60 nm) with a Reichert Ultracut S, mounted on 200 mesh grids, counterstained with 2% uranyl acetate and lead citrate, and observed by using a JEOL 100 CX electron microscope.

Results and Discussion

The segregation of live tumor-derived endothelial cells from other tumor-associated cells, particularly rapidly dividing tumor cells and fibroblasts, precluded methods of tissue explant outgrowth or capillary fragment culture that are successful for microvessel growth for other tissue types (19). Similarly, the diminished level of organization of the tumor microvasculature negated the usefulness of collagenase perfusion for endothelial cell isolation without contamination by tumor cells. Inherent in our approach was the maintenance of characteristics that may be specific to tumor-derived endothelial cells; therefore, methods were utilized to obtain fresh or early passage endothelial cells to minimize potential phenotypic drift of the endothelial cells from the *in vivo* tumor microenvironment.

For endothelial cells to modulate vascular smooth muscle, carboxypeptidase ACE converts angiotensin I to the vasoconstrictor angiotensin II and inactivates the vasodilator bradykinin (13). This endothelial cell marker has also been utilized to selectively isolate endothelial cells by FACS by using a monoclonal antibody which specifically binds endothelial cells from a variety of species (16). To examine the ability of antibody to ACE to effectively label tumor-derived murine endothelial cells, RIF-1 tumors were harvested, dissociated, labeled, and sorted for ACE-positive cells. As shown in Fig. 1*A*, dissociated RIF-1 tumor labeled with antibody to ACE resulted in a wide peak with varying intensity of cells with positive fluorescence for ACE. To maximize purity of the population, only cells with the brightest fluorescence intensity were sorted and plated for culture and further analysis. The positive sorted population was >95% positive for ACE and yielded $1–1.5 \times 10^6$ cells from 2.2×10^7 dissociated RIF-1 tumor cells. With 2.7 ± 0.7 (SD) $\times 10^8$ RIF-1 cells/g of dissociated tumor, ACE-positive cells represented 4–7% of the total population of cells or between 1.1 and 1.9×10^7 ACE-positive cells/g of tumor. The negative sorted cells (RIF-1 tumor cells, fibroblasts, etc.) resulted in a single peak with <5% ACE positive. In addition, cultured RIF-1 tumor cells and NIH/3T3 fibroblasts were negative (<5% positive) for ACE (data not shown). As shown in Fig. 1*B*, the ACE-positive sorted cells exhibited morphological features of endothelial cells with a doubling time of 50 h, as compared to 18 h for RIF-1 tumor cells or the ACE-negative sorted cell population. The ACE-positive cells, however, did not exhibit a cobblestone pattern at confluence. This characteristic cobblestone appearance, while associated with endothelial cell cultures, is not a strict morphological observation (20). Macrophages, which are ACE positive (21), were gated out by orthogonal light scatter as well as excluded through lack of growth in cell culture. As previously reported (22, 23), FACS analysis of primary cultured mouse brain endothelial cells, as well as the hemangioendothelioma cells resulted in relatively pure populations of cells with 88 and 97% ACE positive, respectively (histograms not shown). Cell survival throughout the entire procedure was >90% and ACE-positive endothelial cells were available for analysis within 8 h of tumor harvest.

Further identification of the sorted ACE-positive cells involved the use of ac-LDL and the ability of endothelial cells to take up ac-LDL via the scavenger cell pathway of LDL metabolism (14, 15). ac-LDL preferentially labels endothelial cells and to a lesser extent, pericytes, fibroblasts, and smooth muscle cells with no effect on endothelial cell growth rate. Using DiI-ac-LDL, a fluorescently labeled ac-LDL, sorted ACE-positive cells were examined by FACS for their ability to metabolize DiI-ac-LDL. As shown in Fig. 2*a*, the peaks for cells labeled unsorted and sorted were similar with the percentage of positive DiI-ac-LDL cells 83 and 99, respectively, thereby demonstrating that the population of cells was relatively pure. The sorted DiI-ac-LDL-positive cells exhibited characteristics morphologically similar to endothelial cells and the sorted ACE-positive cells (Fig. 1*B* and 2*B*). Cultures maintained positive labeling for both ACE and DiI-ac-LDL through at least 10 cell passages or 5–6 weeks in culture. Gerritsen *et al.* (24) has utilized DiI-ac-LDL to purify endothelial cells from rheumatoid synovium. Attempts to utilize DiI-ac-LDL as a primary step to isolate tumor-derived endothelial cells from RIF-1 tumors resulted in impure populations of cells contaminated with tumor cells (data not shown).

The principal marker for identification of endothelial cells is factor VIII which refers to a complex of related proteins involved in the initiation of blood coagulation and in platelet function (11, 12). Therefore, sorted ACE/DiI-ac-LDL-positive cells were examined for the presence of factor VIII-related antigen by fluorescent microscopy

(Fig. 3). The fluorescence was predominately perinuclear for both the sorted ACE/DiI-ac-LDL-positive cells and the positive control EOMA cells (Fig. 3, *A* and *B*) with low background fluorescence observed for RIF-1 tumor cells (Fig. 3*C*) and no staining in null antibody controls (data not shown).

By electron microscopy, ACE/DiI-ac-LDL-positive cells were found to exhibit defined junctional structures (Fig. 4, *arrows*), and parallel microfilament arrays (Fig. 4, *arrowheads*) with free ribosomes and vesicular tubular membranous structures typical of endothelial cells, furthermore, on occasion structures reminiscent of Weibel-Palade bodies were apparent (Fig. 4, *inset*). Weibel-Palade bodies are characteristic of endothelial cells and are thought to be a storage site of factor VIII-associated antigen (von Willebrand factor) (25).

These results demonstrate that sufficient numbers of murine endothelial cells can be obtained from solid tumor tissue by using ACE binding and FACS analysis. These tumor-derived cells demonstrate characteristics of microvascular endothelial cells and studies are in progress to investigate the potential differences between these cells and normal endothelium. These differences may include the presence of specific markers, altered patterns of cell adhesion molecules, up- or down-regulation of the expression of extracellular matrix proteins, and variable response to cytokines or other growth factors. The ability to isolate fresh tumor-derived endothelial cells within hours of removal from the tumor microenvironment will enable examination of these potential unique characteristics that could be exploited for design of drug delivery and targeting with novel therapeutic agents.

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